Possible mechanism of superoxide formation through redox cycling of plumbagin in pig heart

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Abstract

The purpose of this study is to elucidate the possible mechanism of superoxide formation through redox cycling of plumbagin (PLG) in pig heart. Of four 1,4-naphthoquinones tested in this study, PLG was most efficiently reduced in the cytosolic fraction of pig heart. On the other hand, lawsone (LAS) was little reduced. Thus, whether or not PLG and LAS induce the formation of superoxide anion radical in pig heart cytosol was examined, by using the methods of cytochrome *c* reduction and chemiluminescence. PLG significantly induced the formation of superoxide anion radical, even though LAS had no ability to mediate superoxide formation. PLG was a significant inhibitor for the stereoselective reduction of 4-benzoylpyridine (4-BP) catalyzed by tetrameric carbonyl reductase (TCBR) in pig heart cytosol. Furthermore, PLG was confirmed to competitively inhibit the 4-BP reduction, and the optimal pH for the PLG reduction was around 6.0 similar to that for the 4-BP reduction. These results suggest that PLG mediates superoxide formation through its redox cycling involved in the two-electron reduction catalyzed by TCBR, and induces oxidative stress in pig heart.

Keywords: Plumbagin; Superoxide formation; Tetrameric carbonyl reductase; Pig heart

1. Introduction

1,4-Naphthoquinones such as menadione (2-methyl-1,4-naphthoquinone, vitamin K_3) are known to cause a variety of toxic effects including cytotoxicity (Babich and Stem, 1993; Klaus et al., 2010). The toxic effects of 1,4-naphthoquinones result from one-electron reduction by mitochondrial NADH-dehydrogenase (NADH:ubiquinone oxidoreductase) and microsomal NADPH-cytochrome P450 reductase, yielding the corresponding semiquinones. The semiquinone participates in redox cycling to generate superoxide anion radical. Unlike mitochondrial NADH-dehydrogenase and microsomal NADPH-cytochrome P450 reductase, cytosolic DT-diaphorase [NAD(P)H:quinone oxidereductase 1 (NQO1)] and carbonyl reductase have the ability to reduce 1,4-naphthoquinones to the corresponding hydroquinones through two-electron transfer mechanism. The resulting hydroquinones are served as substrates for conjugation reactions such as glucuronidation and sulfation. Thus, it is reasonable to assume that these cytosolic enzymes play a role in protection against toxic effects of 1,4-naphthoquinones (Chiou and Tzeng, 2000).

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone, PLG), a naturally occurring 1,4-naphthoquinone isolated from the roots of medicinal plant *Plumbago zeylanica* L., has long been used for various ailments as Indian traditional medicine for than 2500 years. Recently, PLG has been demonstrated to exert anticancer and antiproliferative activities in animal models and in cell cultures (Sandur et al., 2006; Aziz et al., 2008), suggesting that PLG is an excellent lead compound in discovery process of anticancer drugs (Nazeem et al., 2009). However, since the chemical structure of PLG is similar to that of menadione (Fig. 1), which is known to induce cardiotoxicity through the

generation of reactive oxygen species (Chiou et al., 1997), it is possible that PLG also induces cardiotoxicity. In fact, PLG has been reported to generate reactive oxygen species (Inbaraj and Chignell, 2004). Therefore, it is necessary to examine in detail whether or not PLG induces cardiotoxicty.

We have recently purified a tetrameric carbonyl reductase (TCBR) from the cytosolic fraction of pig heart, using 4-benzoylpyridine (4-BP) as the substrate (Usami et al., 2003). TCBR can also catalyze the two-electron reduction of 1,4-naphthoquinones such as menadione (Usami et al., 2003), suggesting that the enzyme catalyzes the two-electron reduction of PLG. The two-electron reduction of PLG catalyzed by TCBR is generally thought to be a detoxification pathway. That is, the resulting hydroquinone can be served as a substrate for glucuronidation and sulfation reactions, as described above. However, Brown et al. (1991) have shown that when these conjugation reactions become limiting, hydroquinones are auto-oxidized to semiquinones. In the heart, the presence of enzymes responsible for conjugation reactions is unknown. Thus, the two-electron reduction of PLG catalyzed by TCBR may subsequently involve redox cycling in the heart. The purpose of this study is to elucidate the possible mechanism of superoxide formation through redox cycling of PLG in pig heart.

2. Materials and methods

2.1. Materials

The chemicals and proteins were purchased from the following sources. PLG, menadione, 4-BP, dicumarol [3,3'-methylenebis(4-hydroxycoumarin)], cytochrome *c*, superoxide dismutase (SOD, from bovine erythrocyte), glucose-6-phosphate dehydrogenase, glucose-6-phosphate, β-NADPH and β-$NADP^+$ were from Wako Pure Chemical Industries (Osaka, Japan); lawsone was from Sigma-Aldrich (St. Louis, MO, USA); 1,4-naphthoquinoe was from Nacalai Tesque (Kyoto, Japan); *S*(−)-α-phenyl-4-pyrodylmethanol [*S*(-)-PPOL], a reduction metabolite of 4-BP, was synthesized from 4-BP as reported previously (Shimada et al. 2003); 2-methyl-6-*p*-methoxyphenylethyl-imidazopyrazine (MPEC, chemiluminesence probe) was from ATTO Corp. (Osaka, Japan). All other chemicals were of reagent grade.

2.2. Preparation of pig heart cytosol

The pig hearts were supplied from a slaughterhouse and stored at –20˚C. The tissues were homogenized in three volume of 10 mM sodium potassium phosphate buffer containing 1.15% KCl (pH 6.0). The homogenate was centrifuged at 105,000 g for 60 min to obtain the cytosolic fraction.

2.3. Reduction of 1,4-naphthoquinones

The reduction of 1,4-naphthoquinones was measured spectrophotometrically by monitoring the decrease of NADPH at 340 nm. The reaction mixture consisted of substrate (1,4-naphthoquinone, menadione, PLG or LAS), 0.3 mM NADPH, pig heart

cytosol and 100 mM sodium potassium phosphate buffer (pH 6.0) in a final volume of 0.5 ml. In the case of determination of the optimal pH, 100 mM sodium potassium phosphate buffers at pH 5.0-9.0 were used. In the case of inhibition experiment by dicumarol at a concentration of 4 μM, PLG was used as the substrate. The enzyme reaction was initiated by the addition of substrate at various concentrations to the reaction mixture. The kinetic parameters (K_m and V_{max}) of enzyme reaction for 1,4-naphthoquinones were analyzed using Lineweaver-Burk plots. One unit of enzyme activity was defined as the amount catalyzing the oxidation of 1 μmol of NADPH/min at 37˚C. The protein concentration was determined with bovine serum albumin as the standard by the method of Lowry et al. (1951).

2.4. Determination of superoxide anion radical by cytochrome c reduction method

Superoxide anion radical was determined by the method of McCord and Fridovich (1969) using cytochrome *c*. The reaction mixture consisted of NADPH-generation system (50 μM $NADP^+$, 1.25 mM glucose-6-phosphate, 50 units glucose-6-phosphate dehydrogenase and 1.25 mM $MgCl_2$), 0.1 mM EDTA, 50 μM cytochrome *c*, pig heart cytosol and 100 mM sodium potassium phosphate buffer (pH 6.0) in a final volume of 1.0 ml. The reaction was started by the addition of PLG or LAS at a concentration of 20 μM. The reduction of ferricytochrome *c* (Fe^{3+}) to ferrocytochrome *c* (Fe^{2+}) in the enzyme reaction system was measured by recording the absorbance at 550 nm.

2.5. Determination of superoxide anion radical by chemiluminescence method

Superoxide anion radical was determined using MPEC-superoxide anion radical kit (ATTO Corp., Osaka, Japan) according to manufacturer's instructions. The reaction

mixture consisted of 10 μM MPEC, NADPH-generation system, pig heart cytosol and 100 mM sodium potassium phosphate buffer (pH 6.0) in a final volume of 1.0 ml. The reaction mixture was incubated with PLG (10 μM) or LAS (10 μM). The light emission was measured using luminescencer PSN AB-2200 (ATTO Corp., Osaka, Japan). The total light emitted was recorded for 60 sec counting period for each tube. The relative luminescence intensity (relative chemiluminesence) was defined as the ratio of luminescence intensity against the background without PLG or LAS (Yamamoto et al., 2005).

2.6. Stereoselective reduction of 4-BP

The stereoselective reduction of 4-BP was estimated by measuring *S*(-)-PPOL formed in pig heart cytosol (Shimada et al., 2004). The reaction mixture consisted of substrate (500 μM 4-BP), NADPH-generation system, pig heart cytosol and 100 mM sodium potassium phosphate buffer (pH 6.0) in a final volume of 0.5 ml. The reaction mixture was incubated at 37˚C for 10 min and boiled for 2 min to stop the reaction. After centrifugation at 5,000 rpm, the supernatant (20 μl) was subjected to HPLC for the determination of *S*(-)-PPOL. HPLC was carried out using a Tosoh DP-8020 HPLC apparatus (Tosoh, Tokyo, Japan) equipped with a Daicel Chiralpak AD-RH column (Daicel, Tokyo, Japan) and a Tosoh UV-8020 monitor (254 nm). Mixture of 20 mM borate buffer (pH 9.0)-acetonitrile (6:4, v/v) was used as a mobile phase at a flow rate of 0.5 ml/min.

2.7. Kinetic analysis

The inhibition mechanism of 4-BP reduction by PLG was kinetically analyzed using

Lineweaver-Burk plots. The velocity (v) was expressed as nmol/min/mg protein. The protein concentration was determined with bovine serum albumin as the standard by the method of Lowry et al. (1951).

2.8. Statistical analysis

Statistical analysis of data was performed using Student's *t*-test, and $P<0.05$ was considered to be significant.

3. Results

3.1. Kinetic parameters for the reduction of 1,4-naphthoquinones

The kinetic parameters for the reduction of four 1,4-naphthoquinones (Fig. 1) in the cytosolic fraction of pig heart are summarized in Table 1. The V_{max}/K_m values for PLG, menadione and 1,4-naphthoquinone were 7725 ± 1101, 1107 ± 74 and 819 ± 97 munits/mg/mM, respectively. The V_{max}/K_m value for LAS was not determined because its reactivity is much lower.

3.2. PLG- and LAS-mediated reduction of cytochrome c

Whether PLG and LAS induce the formation of superoxide anion radical was examined in the cytosolic fraction of pig heart. The absorbance of cytochrome *c* at 550 nm was increased with the time in the presence of PLG, based on the reduction of ferricytochrome *c* to ferocytochrome *c* (line a in Fig. 2 A). Furthermore, SOD decreased the increased absorbance of cytochrome *c* (line b in Fig. 2A), indicating that PLG mediates the formation of superoxide anion radical in pig heart cytosol. SOD could not fully abolish the increased absorbance of cytochrome *c* at 550 mm. This may be because the semiquinone generated in this reaction system also reduces ferricytochrome *c*, as has been pointed out by Winterbourn (1981). On the other hand, LAS had no ability to increase the absorbance of cytochrome *c* at 550 nm (line a in Fig. 2B).

3.3. Reaction of MPEC with superoxide anion radical generated by PLG and LAS

Figure 3 shows the relative chemiluminescences for the reaction of MPEC with

superoxide anion radical generated by PLG and LAS in the cytosolic fraction of pig heart. PLG significantly increased the relative chemiluminescence and SOD caused a significant decrease in the increased relative chemiluminescence, providing further evidence for superoxide formation through redox cycling of PLG in pig heart cytosol. On the other hand, as expected from the result of cytochrome *c* reduction method, LAS had no effect on the relative chemiluminescence.

3.4. Inhibitory effects of 1,4-naphthoquinones on the stereoselective reduction of 4-BP

The inhibitory effects of 1,4-naphthoquinones on the stereoselective reduction of 4-BP to *S*(-)-α-phenyl-4-pyrodylmethanol [*S*(-)-PPOL] were examined in the cytosolic fraction of pig heart. Among these 1,4-naphthoquinones, PLG was the most significant inhibitor, but LAS was the most poor inhibitor for the stereoselective reduction of 4-BP (Table 2).

3.5. Inhibition mechanism for the stereoselective reduction of 4-BP by PLG

The inhibition mechanism for the stereoselective reduction of 4-BP by PLG was examined in the cytosolic fraction of pig heart. Figure 4 shows Lineweaver-Burk plots for the 4-BP reduction in the absence and in the presence of PLG. PLG was found to competitively inhibit the 4-BP reduction.

3.6. pH dependencies for the reduction of PLG and 4-BP

The pH dependencies for the reduction of PLG and 4-BP were examined in the cytosolic fraction of pig heart. As shown in Fig. 5A, the optimal pH for the PLG reduction was around 6.0. A similar result was observed in the optimal pH for the

4-BP reduction (Fig. 5B).

4. Discussion

In the present study, of 1,4-naphthoquinones including menadione, PLG was found to be most efficiently reduced in the cytosolic fraction of pig heart. On the other hand, LAS was little reduced in the cytosolic fraction of pig heart. Thus, whether or not PLG and LAS induce the formation of superoxide anion radical in pig heart cytosol was examined, by using cytochrome *c* reduction method. The obtained results, as expected, showed that PLG mediates the formation of superoxide anion radical in pig heart cytosol, but LAS has no ability to generate superoxide anion radical. The results of the relative chemiluminescence for the reaction of MPEC with superoxide anion radical generated by PLG and LAS in pig heart cytosol also led us to a similar conclusion.

We have recently reported that the stereoselective reduction of 4-BP to *S*(-)-PPOL is catalyzed by TCBR in the cytosolic fraction of pig heart (Shimada et al., 2004). Of 1,4-naphthoquinones examined in this study, PLG was the most significant inhibitor for the stereoselective reduction of 4-BP to *S*(-)-PPOL in pig heart cytosol, even though LAS was the most poor inhibitor for the 4-BP reduction. In addition, PLG was confirmed to competitively inhibit the 4-BP reduction, indicating that this quinone competes with 4-BP for the substrate-binding site on TCBR and can be reduced by TCBR in pig heart cytosol.

To elucidate further the involvement of TCBR in the reduction of PLG in pig heart cytosol, the pH dependencies for the reduction of PLG and 4-BP were examined. The optimal pH for the PLG reduction was around 6.0 and similar to that for the 4-BP reduction; the optimal pH for 4-BP reduction catalyzed by recombinant TCBR is 5.5 - 6.0 (Usami et al., 2003). Although DT-diaphorase also has the ability to reduce

quinones, this cytosolic enzyme has been pointed out to exhibit maximal reduction activity in the pH range 7-9 (Hasspieler and Di Giulio, 1994). Therefore, it is reasonable to assume that in addition to DT-diaphorase, TCBR is involved in the reduction of PLG in the cytosolic fraction of pig heart. This may be also supported from the fact that the decrease in the absorbance of NADPH at 340 nm during the reduction of PLG could not be completely restored by the addition of dicumarol at a concentration of 4 μM (data not shown), which has been shown to produce complete inhibition of DT-diaphorase (Floreani et al., 2000).

Several studies have demonstrated that carbonyl reductase mediates the redox cycling of various quinones (Jarabak, 1991; Jarabak and Harvey, 1993). For example, Jarabak (1991) has shown that human placental carbonyl reductase catalyzes the redox cycling of menadione through two-electron transfer mechanism. In the present study, PLG was found to mediate the formation of superoxide anion radical in pig heart cytosol. This is because not only DT-diaphorase, but also TCBR present in pig heart catalyze the two-electron reduction of PLG. A proposed model of superoxide formation during the redox cycling of PLB catalyzed by TCBR in pig heart is illustrated in Fig. 6. PLG is reduced to the hydroquinone by TCBR. The hydroquinone reacts with PLG to form semiquinone in a so-called comproportionation reaction (Munday, 2000). The resulting semiquinone may produce initial superoxide anion radical. A trace amount of the initial superoxide anion radical oxidizes the hydroquinone to the semiquinone and H_2O_2. The semiquinone then reduces O_2, producing superoxide anion radical and the original PLG. The superoxide anion radical in turn oxidizes the other hydroquinone.

In the heart, the presence of enzymes responsible for conjugation reactions of

hydroquinone is limited. As the result, it is likely that the redox cycling of PLG proceeds in the heart as described above. Recently, PLG has been noted as a lead compound of anticancer drugs. However, we propose the possibility that PLG induces cardiotoxicity due to superoxide formation through its redox cycling. We are currently investigating the detailed mechanism of superoxide formation including the identification of the hydroquinone generated from PLG.

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Figure legends

Fig. 1. Chemical structures of 1,4-naphthoquinones.

Fig. 2. PLG- and LAS-mediated reduction of cytochrome c in pig heart cytosol. (A) line a: PLG (20 μM), line b: PLG (20 μM) + SOD (300 units/ml), line c: control (without PLG). (B) line a: LAS (20 μM), line b: LAS (20 μM) + SOD (300 units/ml), line c: control (without LAS) .

Fig. 3. Relative chemiluminescence for the reaction of MPEC with superoxide anion radical generated by PLG and LAS in pig heart cytosol. The reaction mixture was incubated with MPEC and PLG (10 μM) or with MPEC and LAS (10 μM) in the absence or in the presence of SOD (300 units/ml). The reaction mixture in control was incubated with MPEC alone. Each bar represents the mean ± S.D. of three to eight experiments. *Significantly different from control (P<0.01). #Significantly different from PLG alone (P<0.01).

Fig. 4. Lineweaver-Burk plots for the reduction of 4-BP in the absence and in the presence of PLG. ○, in the absence of PLG; ●, in the presence of PLG (20 μM). Each point represents the mean ± S.D. of three or four experiments.

Fig. 5. pH dependency for the reduction of PLG and 4-BP in pig heart cytosol. PLG (A) and 4-BP (B) at a concentration of 50 μM were used as the substrate. Each point represents the mean ± S.D. of three experiments.

Fig. 6. Proposed model of superoxide formation during the redox cycling of PLG catalyzed by TCBR in pig heart.